Thrombin Induces Proteinase-activated Receptor-1 Gene Expression in Endothelial Cells via Activation of G\textsubscript{i}-linked Ras/Mitogen-activated Protein Kinase Pathway*

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We addressed the mechanisms of restoration of cell surface proteinase-activated receptor-1 (PAR-1) by investigating thrombin-activated signaling pathways involved in PAR-1 re-expression in endothelial cells. Exposure of endothelial cells transfected with PAR-1 promoter-luciferase reporter construct to either thrombin or PAR-1 activating peptide increases the steady-state PAR-1 mRNA and reporter activity, respectively. Pretreatment of reporter-transfected endothelial cells with pertussis toxin or co-expression of a minigene encoding 11-amino acid sequence of COOH-terminal G\textsubscript{i} prevented the thrombin-induced increase in reporter activity. Pertussis toxin treatment also prevented thrombin-induced MAPK phosphorylation, indicating a role of G\textsubscript{i} in activating the downstream MAPK pathway. Expression of constitutively active G\textsubscript{q} or G\textsubscript{q12} subunits increased reporter activity 3–4-fold in the absence of thrombin stimulation. Co-expression of dominant negative mutants of either Ras or MEK1 with the reporter construct inhibited the thrombin-induced PAR-1 expression, whereas constitutively active forms of either Ras or MEK1 activated PAR-1 expression in the absence of thrombin stimulation. Expression of dominant negative Src kinase or inhibitors of phosphoinositide 3-kinase also prevented the MAPK activation and PAR-1 expression. We conclude that thrombin-induced activation of PAR-1 mediates PAR-1 expression by signaling through G\textsubscript{q12} coupled to Src and phosphoinositide 3-kinase, and thereby activating the downstream Ras/MAPK cascade.

Endothelial cell responsiveness to thrombin requires the expression of cell surface proteinase-activated receptor-1 (PAR-1)

1 (1–6). Activation of PAR-1 by cleavage of the NH\textsubscript{2} terminus

of PAR-1 results in changes in endothelial cell function such as expression of endothelial cell adhesion molecule ICAM-1 and increased endothelial permeability (7–9). The regulatory mechanisms underlying PAR-1 gene expression are not well characterized. Thrombin-induced PAR-1 cleavage leads to PAR-1 endocytosis and degradation in lysosomes (10); therefore, the activated PAR-1 is unable to recycle to the cell membrane and resestizes cells to thrombin as with other G protein-coupled receptors (10). Previous studies have shown that endothelial cells also contain a preformed pool of PAR-1 that translocates within minutes to the cell surface in response to thrombin (10, 11). This protein synthesis-independent PAR-1 expression confers thrombin sensitivity by rapidly replenishing the cell surface PAR-1 (10); however, this pool has a limited and short-lived ability to resestize endothelial cells to thrombin (5).

Further, thrombin responsiveness required de novo PAR-1 synthesis (5), indicating that it is necessary to activate synthesis of PAR-1 to regenerate fully the cell surface PAR-1 population once the preformed receptor pool has been depleted.

PAR-1 has been shown to couple functionally with multiple heterotrimeric G proteins, which may in part explain the pleotropic actions of thrombin (12, 13). In endothelial cells, PAR-1 binds to heterotrimeric G\textsubscript{i} and G\textsubscript{q}, and can thus initiate downstream signaling events (13). As receptors coupled to G\textsubscript{i} activate the Ras/MAPK cascade, which in turn results in gene transcription (14–18), in the present study, we investigated the possible role of G\textsubscript{i} activation of Ras/MAPK pathway in regulating thrombin-induced PAR-1 gene expression in endothelial cells.

**EXPERIMENTAL PROCEDURES**

Materials—Dulbecco’s modified Eagle’s medium (DMEM) and diethyl pyrocarbonate were purchased from Sigma. Fetal bovine serum (FBS) was obtained from HyClone, Logan, UT. Duralose membrane, Quickhyb, and Prime-It II were from Stratagene, La Jolla CA. PAR-1 activating peptide, SPLLRFNPNDKYPF (TRP-14) was synthesized and purified as described (4). An ~3-kb 5′-regulatory portion of cloned PAR-1 gene (19, 20) subcloned into pBluescript was obtained from Dr. W. F. Bahou (SUNY, Stony Brook, NY). TRIzol reagent, polymerase chain reaction (PCR) primers, green fluorescent protein (Green Lantern-1) plasmid DNA, LipofectAMINE, and Opti-MEM I were from Life Technologies Inc.. The reporter vectors pGL2 (firefly, Photinus pyralis) and pRL (sea panay, Renilla reniformis) luciferase and the Dual Luciferase Reagent Assay System were from Promega Corp., Madison, WI. Protein assay reagents were from Bio-Rad. Genistein, 4-amino-5-(4-
methyl-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1), wortmannin, LY294002, and anti-v-Src antibody were from Calbiochem-Novabiochem, La Jolla, CA, Pertussis toxin was from List Biologicals, Campbell, CA. Anti-Erk1, anti-p85 phosphomimetic S-kinase (PI3K), and anti-Shc antibodies were from Transduction Laboratories, Lexington, KY. The anti-hemagglutinin receptor monoclonal antibody E1.7.3 was from Avusa Limited, Hertfordshire, UK. Phosphorylated MAP kinase substrate (22A) was from Promega Corp. Plasmid DNA encoding mutant forms of MEK (HA-tagged MKK1 in pCEP4), CAAX-Raf (in pCEX3V), Goa (205L in pcDNA1), Gβ1 (in pcDNA3.1), Gγ2 (in pcDNA3.1), bovine GRIK2 (in pcDNA3.0), and α subunit of bovine retinal transducin (in pcDNA1.0) were from Dr. T. Voyno-Yasenetskaya (University of Illinois, Chicago, IL). These expression constructs were prepared as described (21). Expression plasmids encoding Gαi2 minigene antagonists (coding sequence 5'-ATCAAGAAGCTG-AAGACTGGGGCTTCC-3' in pcDNA3) and mi geneine with scrambled sequence (coding sequence 5'-ATGGGAAAGCGTACGTTCTCTCA-ACGACAAGGCT-3' in pcDNA3) were from Dr. H. E. Hamm (Northwestern University, Chicago, IL). The complimentary oligonucleotides were synthesized and annealed at 65 °C, and the annealed double-strand DNA was ligated in pcDNA3.1 vector (22). The wild-type c-Src and dominant negative mutant c-Src (K295MY527F) in pSM vector were obtained from Dr. S. Gutkind (NIDR, National Institutes of Health, Bethesda, MD). Ras mutants cloned into pMAMneo expression vector were from Dr. P. Sassi (University of Illinois, Chicago, IL). Electrodes for endothelial cell monolayer resistance measurements were purchased from Applied Biophysics, Inc., Troy, NY.

Construction of PAR-1 Reporter Plasmid—A 1.82-kb PAR-1 promoter fragment was obtained by PCR amplification of directly upstream of the initiation codon from an HMEC, grown in six-well plates to 50–70% confluence. Endothelial cell lysates or immunoprecipitates were treated with or without agonist for the indicated times at 37 °C. Cells were washed three times with PBS and saline and their protein content was measured by the Bradford assay. HMEC were lysed (according to manufacturer’s instructions) and 1 mg/ml hydrocortisone. Human pulmonary artery endothelial cell line (HMEC) were obtained from Dr. T. Voyno-Yasenetskaya (University of Illinois, Chicago, IL). These cell lines were from Transduction Laboratories, Lexington, KY. The wild-type c-Src and dominant negative mutant c-Src (K295MY527F) in pSM vector were obtained from Dr. S. Gutkind (NIDR, National Institutes of Health, Bethesda, MD). Ras mutants cloned into pMAMneo expression vector were from Dr. P. Sassi (University of Illinois, Chicago, IL). Electrodes for endothelial cell monolayer resistance measurements were purchased from Applied Biophysics, Inc., Troy, NY.

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serum-free medium and incubated for 2 h in 1% serum-supplemented culture medium. The small and large electrodes were connected to a phase-sensitive lock-in amplifier. A constant current of 1 μA was applied by a 1-V, 4000-Hz AC connected serially to a 1-megohm resistor between the small electrode and the larger counter electrode. The voltage change between small electrode and larger counter electrode was continuously monitored by lock-in amplifier, stored, and processed on a computer. The data are presented as change in resistive (in-phase) phase-sensitive lock-in amplifier. A constant current of 1 μA was applied by a 1-V, 4000-Hz AC connected serially to a 1-megohm resistor between the small electrode and the larger counter electrode. The voltage change between small electrode and larger counter electrode was continuously monitored by lock-in amplifier, stored, and processed on a computer. The data are presented as change in resistive (in-phase) portion of the impedance normalized to its initial value at time zero as described (25, 26).

**Statistical Analysis**—Statistical comparisons were made using two-tailed Student’s t test. Experimental values were reported as mean ± S.E. Differences in mean values were considered significant at p < 0.05.

**RESULTS**

**Thrombin-induced PAR-1 Activation Increases Steady-state PAR-1 mRNA**—To determine if PAR-1 activation increases the PAR-1 mRNA levels in endothelial cells, Northern blot analysis was performed on total RNA isolated from agonist-stimulated cells (see “Experimental Procedures”). Confluent endothelial cell monolayers were stimulated for the indicated times with PAR-1 agonists, thrombin, or the 14-amino acid PAR-1 tethered ligand, TRP-14 (12). The expression of GAPDH was unaffected by the experimental conditions and was thus used as an internal control. In primary cells, the HPAEC, PAR-1/gapdh ratio increased 2-fold (over untreated controls) after thrombin treatment (Fig. 1A). This increase was sustained through 6 h of thrombin treatment. A similar response was observed with thrombin treatment of HMEC (Fig. 1B). Stimulation of HPAEC with TRP-14 also increased PAR-1/GAPDH 1.9-fold by 6 h (Fig. 1C).

**Thrombin-mediated Transactivation of PAR-1 Promoter**—To explore the induction of PAR-1 gene, the PAR-1 promoter reporter plasmid (PAR-1/Luc) and TK/pRL (see “Experimental Procedures”) were transiently expressed in HMEC. Cells were challenged with either thrombin or TRP-14 for various times and lysed, and luciferase activity was measured (see “Experimental Procedures”). Luciferase activity (relative light unit ratio (RLU/mg of protein) was expressed after subtracting the basal activity at each time point. Black columns, 100 nM thrombin treatment; white columns, 100 mM TRP-14 treatment. Time course where each point (shown as mean ± S.E.) represents three to six wells per experiment. Experiments were performed four times.

**Fig. 1. Thrombin induces PAR-1 gene transcription.** Endothelial cells grown to confluence were stimulated with thrombin or TRP-14 for the indicated times prior to isolation of total cellular RNA and Northern blot analysis (described under “Experimental Procedures”). A, HPAEC stimulated with 25 nM thrombin. B, HMEC stimulated with 100 nM thrombin. C, HPAEC stimulated with 25 mM TRP-14. Band intensity was quantified using NIH Image version 1.6, and ratios of PAR-1 to GAPDH plotted for each autoradiogram are shown. Results are representative of two to five experiments per group.

**Fig. 2. PAR-1-driven luciferase expression by activation of PAR-1.** HMEC grown in six-well plates, transfected with the PAR-1/Luciferase (300 ng) and TK/pRL (20 ng) reporter plasmids, were stimulated with 100 nM thrombin or 100 mM TRP-14 and lysed at indicated times. Firefly and sea pansy luciferase activity was measured from 20 μl of total cell lysate (described under “Experimental Procedures”). Luciferase reporter activity (relative light unit ratio (RLU/mg of protein) was expressed after subtracting the basal activity at each time point. Black columns, 100 nM thrombin treatment; white columns, 100 mM TRP-14 treatment. Time course where each point (shown as mean ± S.E.) represents three to six wells per experiment. Experiments were performed four times.

**Fig. 3. Thrombin-mediated induction of PAR-1-driven luciferase activity is pertussis toxin-sensitive.** HMEC were transfected with luciferase-reporter plasmids (described under “Experimental Procedures”) and pretreated overnight with pertussis toxin (100 ng/ml). HMEC were then stimulated with 100 nM thrombin for 4 h and lysed, and luciferase activity was measured. RLU were corrected for milligrams of total protein. Luciferase activity (mean ± S.E.) from HMEC -fold increase is [(RLU/mg protein treated)/(RLU/mg protein untreated)]. Black columns, 100 nM thrombin treatment; white columns, without thrombin treatment. The mean ± S.E. from four experiments repeated in triplicate are shown. Asterisk (*) indicates the difference from thrombin-stimulated control (p < 0.005).
activity increased >2.5-fold over basal after 4 h of 100 nM TRP-14 stimulation, indicating that activation of endothelial cell surface PAR-1 is responsible for the induction of PAR-1 gene expression.

**Pertussis Toxin-sensitive G Proteins Mediate Thrombin-induced PAR-1 Gene Expression**—To determine the role of G, in thrombin-mediated PAR-1 gene induction, HMEC expressing the reporter construct were pretreated with pertussis toxin (PTX) to uncouple PAR-1 from PTX-sensitive G proteins. PTX pretreatment prevented the increase in luciferase activity by 90%, following thrombin stimulation (Fig. 3). Basal luciferase levels were not altered by PTX pretreatment. Thus, PTX-sensitive G proteins are critically involved in mediating thrombin-induced PAR-1 gene expression.

The interface between the Ga subunits of heterotrimeric G proteins and cytosolic domains of heptahelical receptors are potential targets for interrupting agonist-mediated signaling events (23). To address further the role of Gi, we determined whether a Ga-peptide antagonist interferes with thrombin-mediated PAR-1 expression. We transfected plasmids encoding a peptide corresponding to the COOH terminus of Ga to disrupt the G protein-receptor interaction (23). Expression of the Ga antagonist peptide inhibited thrombin-induced PAR-1 expression (Fig. 4A). Overexpression of a scrambled peptide of the same amino acid composition did not alter luciferase expression following thrombin treatment. These results combined with the inhibitory effect of PTX on PAR-1 gene expression indicate the involvement of G,-PAR-1 coupling in mediating thrombin-induced PAR-1 gene induction.

Receptors coupled to PTX-sensitive Gi proteins have been shown to activate downstream signal transduction pathways in both a and bg subunit-dependent manner (14, 15, 27). To address if a or bg subunits coupling to PAR-1 is responsible for downstream signaling, we co-expressed a constitutively active Ga, mutant (G,-Q205L) or bg subunits with reporter construct in HMEC. Expression of mutant Ga, increased reporter activity 3–4-fold over basal values (Fig. 4B), suggesting the involvement of PTX-sensitive G proteins in the thrombin-induced PAR-1 gene induction. Co-expression of plasmids encoding the Gbg1- and Gbg2-isofoms along with luciferase-reporter construct induced PAR-1 gene expression in the absence of thrombin treatment (Fig. 4C). However, neither Gbg1 nor Gbg2 alone induced PAR-1 gene expression. Further, we co-expressed the Gbg2 sequesters such as G protein-coupled receptor kinase 2 (GRK2) and transducin with the luciferase-reporter construct (28, 29). Co-expression of either GRK2 or transducin assayed for luciferase activity. Black columns, 4 h of 100 nM thrombin treatment; white columns, without thrombin treatment. Results are mean ± S.E. from five experiments. Asterisk (*) indicates the difference from thrombin-stimulated control (p < 0.005). B, constitutively active Ga, mutant (G,-Q205L) induces PAR-1 expression. HMEC were co-transfected with the reporter plasmids (as described in Fig. 2) and 100 ng of G,-Q205L for 4 h. Cells were lysed and assayed for luciferase activity. Black columns, 4 h of 100 nM thrombin treatment; white columns, without thrombin treatment. Values are shown as mean ± S.E. from four experiments made in triplicate. Asterisk (*) indicates the difference from basal expression (p < 0.001). C, Gbg expression in HMEC activates PAR-1 expression. Plasmids encoding Gbg1 or Gbg2 were co-transfected with the luciferase-reporter plasmids in HMEC. Black columns, 4 h of 100 nM thrombin treatment; white columns, without thrombin treatment. Values are shown as mean ± S.E. from four experiments of three wells in each group. Asterisk (*) indicates difference from basal reporter activity (p < 0.005). D, Gbg scavenger co-expression prevents thrombin-induced PAR-1 expression. Plasmids encoding GRK2 or transducin were co-transfected with the luciferase-reporter plasmids in HMEC. Black columns, 4 h of 100 nM thrombin treatment; white columns, without thrombin treatment. Values are shown as mean ± S.E. from four experiments of three wells in each group.
Ras-activated Pathway Mediates Thrombin-induced PAR-1 Expression—To address the role of Ras and Ras-activated pathway in thrombin-induced PAR-1 gene expression, luciferase-reporter constructs were co-expressed with dominant-negative Ras (N17Ras) in HMEC. In N17Ras-expressing HMEC, PAR-1-driven luciferase activity was reduced by 51% following thrombin treatment (Fig. 6A). Moreover, the oncogenic form of Ras, V12Ras, activated PAR-1 gene transcription independent of stimulation by thrombin (Fig. 6A).

Since an immediate downstream target of Ras is the serine/threonine kinase Raf, we tested the ability of Raf kinase to recruit PAR-1 gene transcription. In this experiment, the luciferase activity increased >2-fold in HMEC overexpressing CAAX-Raf (Fig. 6A). As a known substrate for Raf kinase is the MAPK kinase, MEK1, the upstream activator of MAPK, we also determined the effects of overexpression of dominant-negative MEK1. These results indicated that the dominant-negative MEK1 abolished the thrombin-induced PAR-1 gene expression (Fig. 6B). However, the oncogenic MEK1 induced the PAR-1-driven luciferase activity (1.8-fold over untreated) inde-
dependent of thrombin stimulation, a response similar to the constitutively active mutants of Ras and Raf. Western blot analysis using an anti-hemagglutinin polyclonal antibody demonstrated the relative expression of mutant MEK1 isosforms in HMEC (Fig. 6B, inset). Thus, the Ras/Raf/MAPK signaling pathway is critical in mediating the thrombin-induced expression of PAR-1 gene in endothelial cells.

Inhibition of Protein-tyrosine Kinase Signaling Pathways Prevents Thrombin-induced PAR-1 Gene Expression—Because tyrosine kinase signaling activates Ras (32), we assessed the role of protein-tyrosine kinase (PTK)-dependent signaling in mediating PAR-1 gene expression in HMEC. Genistein, a PTK inhibitor, was used to investigate PTK-dependent signal transduction. Thrombin-activated MAPK phosphorylation was measured after pretreating HMEC with 30, 100, or 300 μM genistein. We showed that genistein decreased the thrombin-induced MAPK phosphorylation in a dose-dependent manner (Fig. 7A).

To assess the role of PTK-dependent signaling in PAR-1 gene transcription, HMEC transfected with luciferase-reporter plasmids were pretreated with 100 μM genistein for 2 h prior to stimulation with 100 nM thrombin. These results showed that genistein pretreatment inhibited luciferase activity by 80% (Fig. 7B), whereas basal luciferase activity was unaffected by PTK inhibition. Genistein also prevented the thrombin-induced increase in PAR-1 mRNA levels at 3 and 6 h (Fig. 7C), indicating the dependence of PTK signaling in the induction of PAR-1 gene.

Following functional depletion of endothelial cell PAR-1, cellular resensitization to thrombin required 18 h and the response was de novo PAR-1 synthesis-dependent (5). Therefore, we examined a functional consequence of genistein on the protein synthesis-dependent resensitization to thrombin at 18 h. HPAEC were pretreated with 100 μM genistein prior to measuring thrombin-induced cell shape change by the electrical resistance method (25) (see “Experimental Procedures”). The drop in transendothelial electrical resistance (a measure of cell retraction) in response to the initial thrombin concentration, which was sufficient to deplete PAR-1 in HPAEC, was genistein-insensitive (Fig. 8, inset); however, the protein synthesis-dependent cell retraction (i.e. the cycloheximide-sensitive response) occurring at 18 h after thrombin challenge (5) was inhibited 70% by genistein (Fig. 8). Therefore, genistein did not prevent the initial thrombin-induced HPAEC shape change, but it prevented the delayed, cycloheximide-sensitive response following PAR-1 depletion.

Shc Phosphorylation following Thrombin Treatment—The activation of Ras by cell surface receptors involves formation of a Shc-Grb2-SOS1 complex that depends on Src homology 2-mediated recognition of phosphoryrosine (33). Src-mediated tyrosine phosphorylation of Shc is essential for this complex formation (34). As phosphorylation of Shc is critical in such protein-protein interactions (35), we determined the phosphorylation of Shc adapter proteins in HMEC. Phosphorylation of p46, p52, and p66 Shc was detected by anti-Shc immunoblotting following 1–2 min of thrombin exposure (Fig. 9A). Pretreatment of HMEC with Src kinase inhibitor PP1 markedly reduced the thrombin-induced tyrosine phosphorylation of Shc isoforms. Fig. 9B shows the expression of all three isoforms of Shc in HMEC. These results indicate that Src-mediated Shc phosphorylation serves as an upstream signaling intermediate contributing to thrombin-activated Src homology 2-dependent protein interactions. Inhibition of Src Kinase Activity Prevents Thrombin-induced MAPK Phosphorylation and Luciferase Expression—Src kinase is an upstream regulator of Ras (36), and stimulation of Src kinases by G protein-coupled receptors results in tyrosine phosphorylation of Shc and subsequent p21ras activation (34, 37). We used two distinct inhibitors, herbinycin A and PP1, to assess the role of Src family of PTK in thrombin-induced intracellular signaling. Phosphorylation of MAPK in response to 2 min of thrombin exposure was measured in HMEC pretreated with 1, 10, or 30 nM herbinycin A (Fig. 10A). Herbinycin A concentrations as low as 1 nM inhibited thrombin-induced MAPK phosphorylation to levels comparable to unstimulated cells. PP1 (10 μM) also inhibited the thrombin-induced MAPK phosphorylation (data not shown). In addition, pretreatment of HMEC with 10 μM PP1 prevented thrombin-mediated increases in luciferase activity (Fig. 10B). Moreover, co-expression of dominant negative Src (c-Src K295M/Y527F) with the
reporter plasmid prevented the induction of luciferase expression (Fig. 11A). Western blot analysis demonstrated the relative expression of c-Src in each group (Fig. 11B). Thus, Src kinase phosphorylates Shc to activate downstream signals, and signals the thrombin-induced PAR-1 gene expression.

**Phosphoinositide 3-Kinase Activity Is Necessary for Thrombin-induced PAR-1 Gene Expression**—PI3Ks, lipid kinases existing in heterodimeric (PI3Ka) and monomeric (PI3Kγ) forms, have been implicated in G protein-coupled receptor-mediated signaling (38, 39). One isoform, PI3Kγ, is stimulated by Gβγ...
once the pre-formed PAR-1 pool has been depleted (5, 10). Inhibition of protein synthesis by cycloheximide prevented the full recovery of cell surface PAR-1 after thrombin-induced PAR-1 depletion (5), indicating de novo protein synthesis was required for the response. In the present study, we demonstrate that activation of cell surface PAR-1 by thrombin is itself a critical stimulus for PAR-1 gene expression in vascular endothelial cells. We showed in HPAEC and HMEC that PAR-1 activation increased PAR-1 mRNA and PAR-1 promoter-driven luciferase-reporter activity. Activation of PAR-1 was sufficient to increase PAR-1 mRNA since endothelial cells challenged with PAR-1 activating peptide (TRP-14), also induced PAR-1 gene expression. Therefore, the protein synthesis-dependent PAR-1 resensitization in vascular endothelial cells is attributed to the activation of PAR-1 gene expression.

Studies on the effects of thrombin on PAR-1 mRNA in human glomerular mesangial and human erythroblastemia (HEL) cells (41, 42) showed that PAR-1 mRNA was unaffected by thrombin in mesangial cells, whereas PAR-1 mRNA increased within 6 h of thrombin exposure in HEL cells. HEL cells undergo prolonged PAR-1 desensitization following thrombin exposure (43); however, HEL cells, unlike endothelial cells, lack a cytosolic PAR-1 pool (43). Recovery of thrombin sensitivity in HEL cells was associated with new receptor synthesis since cycloheximide prevented the resensitization (43). Results of the present study showing that thrombin activation of PAR-1 in endothelial cells induced PAR-1 gene expression within 3–6 h after thrombin exposure are consistent with the HEL cell experiments (43).

Proteolytic cleavage of the surface receptors PAR-1, PAR-2, PAR-3, and PAR-4 by proteases (i.e. thrombin in the case of PAR-1, PAR-3, and PAR-4 and trypsin or trypsin in the case of PAR-2) unmask a cryptic ligand that in PAR-1 interacts with the extracellular loop 2 (12, 44–47). Binding of the tethered ligand to extracellular loop 2 domain stimulates PAR-1, and generates intracellular second messengers (47). PAR-2 and PAR-3 have been shown to be expressed in human umbilical vein endothelial cells (48, 49). PAR-2 is trypsin-specific receptor, but it can also be activated by PAR-1 activating peptide (TRP-14) (50). PAR-3 and PAR-4 cannot be activated by TRP-14 (45, 46). In the present study, we were unable to show that a functionally active PAR-2 in HPAEC since the PAR-2-specific activating peptide (SLIGKV) did not induce either cell retraction or increase in [Ca$^{2+}$], (data not shown). Therefore, the activation of TRP-14-induced PAR-1 gene expression can only be ascribed to stimulation of PAR-1.

Thrombin activates signaling in endothelial cells by coupling to PAR-1 to heterotrmeric $G_i$ and $G_q$ (13, 51). Our results using PTX to address the role of heterotrmeric $G_i$ in activating PAR-1 gene showed that thrombin-induced PAR-1 gene transcription was PTX-sensitive. To explore further the role of PTX-sensitive heterotrermic $G$ proteins, a minigene encoding a peptide antagonist homologous to the $G_q$ COOH terminus of $G_\alpha_q$ was co-expressed with the reporter construct in HMEC. We showed that overexpression of this $G_q$ peptide inhibited thrombin-induced PAR-1 gene expression. Expression of constitutively active mutant of $G_{\alpha_i}$ or $G_{\alpha_i}$ subunits in HMEC was sufficient to transactivate the PAR-1 promoter in absence of thrombin. Further, we showed that co-expression of $G_{\alpha_i}$ subunits sequesters such as GRK2 and transducin with PAR-1 promoter construct prevented thrombin-induced expression of PAR-1 in HMEC (28, 29). These data demonstrate that $G_q$ activation mediates thrombin-induced PAR-1 expression by $G_{\alpha_q}$ dissociation from the ligand-activated $G_{\alpha_q}$.

We measured the activation of MAPK to ascertain MAPK involvement in the signal transduction pathway downstream of

Fig. 12. A, wortmannin and LY294002 inhibit thrombin-induced MAPK phosphorylation. HMEC pretreated for 2 h with wortmannin (30, 100, and 300 nM) (upper) or LY294002 (30, 100, and 300 nM) (lower) were assayed for MAPK phosphorylation following 2 min of 100 nM thrombin stimulation. Blots were stripped and reprobed with anti-Erk1 antibodies. Phospho-MAPK positive control (*). B, PI3K activity is required for thrombin-induced PAR-1 gene expression. HMEC transfected with luciferase-reporter constructs were pretreated with 100 nM wortmannin or 100 nM LY294002 for 2 h prior to 4 h of thrombin exposure. Cells were lysed, and luciferase activity was measured. Black columns, 4 h of 100 nM thrombin treatment; white columns, without thrombin treatment. Values are shown as mean ± S.E. from four experiments of three to six wells in each group. Asterisk (*) indicates difference from the thrombin-stimulated control (p < 0.05).
Gαs Phosphorylation of MAPK on threonine and tyrosine residues by MEK is known to stimulate MAPK activity (52). We showed that thrombin maximally activated MAPK phosphorylation within 2 min, and pretreatment of HMEC with PTX prevented the thrombin-induced MAPK phosphorylation. PTX also abolished MAPK-dependent phosphorylation of Elk-1 in an in vitro kinase assay. To delineate the signaling mechanisms conveying the thrombin signal to PAR-1 promoter, we introduced dominant negative (dn) mutants of Ras and MEK1 into HMEC. We showed that overexpressing dn-mutant of Ras or MEK1 interfered with activation of downstream signaling. Expression of dn-Ras (N17Ras) in contrast to dn-MEK1 inhibited PAR-1 gene induction by 50% in response to thrombin challenge. This may be the result of incomplete inhibition of thrombin-mediated Ras activation by N17 Ras expression or alternatively to a Ras-independent signaling mechanism (53).

The finding that dn-MEK1 mutant fully inhibited PAR-1 gene transcription following thrombin stimulation implies that MAPK activity is necessary for PAR-1 gene induction since MEK1 is situated directly upstream of MAPK in the Ras/Raf/MAPK pathway. We also showed that overexpressing the constitutively active mutants of Ras (V12Ras), Raf (CAAX-Raf), and MEK1 (S218E/S222E) resulted in transactivation of the constitutively active mutants of Ras (V12Ras), Raf (C

MEK1 is situated directly upstream of MAPK in the Ras/Raf/MAPK signaling pathway.

We showed that genistein pretreatment prevented MAPK phosphorylation and PAR-1 gene induction, as demonstrated by both reporter assay and mRNA blotting. Genistein has been shown to prevent the de novo protein synthesis-dependent resensitization to thrombin following PAR-1 depletion in endothelial cells (5). Thus, thrombin-induced activation of PAR-1 promoter relies on the PTK-dependent signaling pathway.

P38K and Src-like tyrosine kinases lie between Gαs and the downstream Ras/MAPK signaling cascade (14, 15). We showed that inhibition of Src activity by PP1 prevented the induction of PAR-1 gene in response to thrombin. In addition, overexpression of dn-Src abrogated PAR-1 gene induction, whereas wild type Src had no effect on thrombin signaling. Previous studies have shown that PTK activity in the formation of Shc-Gβ2-SOS1, which in turn activates p21ras (14, 15), and phosphorylation of Shc on tyrosine residues by Src kinase is a prerequisite for Grb2 association and subsequent SOS1 recruitment (34). We showed that thrombin stimulated the rapid tyrosine phosphorylation of Shc adapter proteins and that this was inhibited by Src kinase inhibitor PP1, suggesting that Src can activate Ras/MAPK via this mechanism, and thus signal PAR-1 expression. Thrombin-induced MAPK phosphorylation and PAR-1 gene expression were sensitive to P38K inhibition by both wortmannin and LY294002. The p85 subunit of P38K contains a Src homology 2 domain interacting with Shc-Grb2 complex (39, 40), suggesting that activation of P38K can lead to activation of the downstream PTK pathway, and thereby to PAR-1 gene expression. The present data are consistent with the hypothesis that Gαi released from Gβγ activates Src-like PTK and P38K, and this in turn activates Ras/MAPK signal transduction, and induces PAR-1 gene expression in endothelial cells. In summary, the present data indicate that thrombin-mediated PAR-1 gene expression in endothelial cells requires the heterotrimeric Gαi-activated Ras/MAPK signaling pathway. The pathway linking Gαi to Ras relies on P38K and Src-like PTK activity as well as Shc-Grb2-SOS1 complex formation. We propose that thrombin-induced PAR-1 gene activates the Gαi-linked Ras/MAPK cascade resulting in transactivation of the PAR-1 promoter and PAR-1 expression.
PAR-1 Gene Expression in Endothelial Cells

Thrombin Induces Proteinase-activated Receptor-1 Gene Expression in Endothelial Cells via Activation of G_{i}-linked Ras/Mitogen-activated Protein Kinase Pathway
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